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Interleukin 20: Discovery, Receptor Identification, and Role in Epidermal Function

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Summary

A structural, profile-based algorithm was used to identify interleukin 20 (IL-20), a novel IL-10 homolog. Chromosomal localization of IL-20 led to the discovery of an IL-10 family cytokine cluster. Overexpression of IL-20 in transgenic (TG) mice causes neonatal lethality with skin abnormalities including aberrant epidermal differentiation. Recombinant IL-20 protein stimulates a signal transduction pathway through STAT3 in a keratinocyte cell line, demonstrating a direct action of this ligand. An IL-20 receptor was identified as a heterodimer of two orphan class II cytokine receptor subunits. Both receptor subunits are expressed in skin and are dramatically upregulated in psoriatic skin. Taken together, these results demonstrate a role in epidermal function and psoriasis for IL-20, a novel cytokine identified solely by bioinformatics analysis.

Introduction

We describe the use of EST database mining in the discovery of interleukin-20 (IL-20), a novel IL-10-related cytokine. The IL-10 protein family includes IL-10 (Vieira et al., 1991), IL-19 (Rosen and Kenny, 1999), MDA-7 (Jiang et al., 1995), IL-TIF (Dumoutier et al., 2000a), and AK-155 (Knappe et al., 2000). The biological function of IL-10 itself has been explored in depth. IL-10 mediates its activity through a receptor which is composed of IL-10Rα and IL-10Rβ (CRF2-4; Kotenko et al., 1997). These receptor subunits form a heterodimeric structure similar to that found in interferon and other class II cytokine receptors. IL-10 regulates the function of both lymphoid and myeloid cells, and its immunosuppressive effects involve inhibition of proinflammatory cytokine synthesis by T cells, monocytes, and macrophages. IL-10 can also act as an immunostimulant by increasing thymocyte, mast cell, and B cell proliferation (Moore et al., 1993). Potential actions of IL-10 in a variety of cutaneous disorders were recently reviewed, including a therapeutic role for IL-10 as an anti-inflammatory cytokine in psoriasis (Asadullah et al., 1999).

We discovered IL-20 utilizing a bioinformatics algorithm designed to identify helical cytokines. Using a multifaceted approach, we have elucidated a biological function and disease association for the IL-20 protein. Results from TG overexpression of IL-20 in mice and in vitro assays using recombinant IL-20 protein led us to the discovery of an IL-20 receptor. This heterodimeric receptor is structurally related to the IL-10 receptor. We describe the biological activity of IL-20 in skin and show its role in psoriasis appears to be opposite to that of IL-10.

Results

Identification, Cloning, and Structural Analysis of IL-20

EST databases were searched using an algorithm designed to identify translated sequences containing both a signal sequence and one or more amphipathic helices commonly found in helical cytokines. One of the highest scores was from a single EST (INC819592) found in a human keratinocyte library (Incyte Genomics, Inc.). Based on the EST sequence, nested oligonucleotides were designed and 3′ RACE (rapid amplification of cDNA ends) was performed on RNA isolated from human skin and trachea, yielding the remainder of the coding sequence, 3′ untranslated region (UTR) and polyadenylation sequence. The 3′ UTR of this cDNA contains seven AUUUA and one perfect UUAUUUAUU mRNA instability motifs (Zubiaga et al., 1995) often found in cytokine mRNAs, which may explain its rarity in EST databases. Northern analysis indicates that this mRNA is expressed at very low levels in skin, trachea and in other tissues (data not shown).

Analysis of the entire coding sequence not only supports the classification of this protein as a helical cytokine, but also indicates that it is a member of the IL-10 family. This novel cytokine was named interleukin 20 (IL-20). Figure 1A shows a sequence comparison between human IL-20 and its three closest relatives based on amino acid sequence identity: IL-19 (40%), MDA-7 (33%), and IL-10 (28%). IL-20 also shares sequence identity with two other IL-10 family members, IL-TIF (25%) and AK-155 (24%). Mouse IL-20 cDNA was isolated from a mouse skin library using a human IL-20 coding region probe. Both human and mouse IL-20 contain 176 amino acids and are 76% identical in amino acid sequence.

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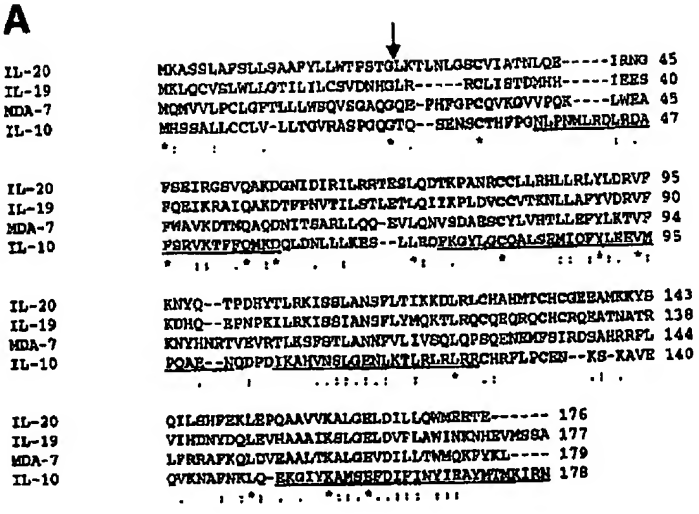


Figure 1. Multiple Alignment and Chromosomal Mapping of the IL-10 Protein Family

(A) Sequence of human IL-20 and its alignment with three other human members of the IL-10 protein family. Asterisks denote conserved residues, colons represent highly conserved residues, dots represent conservative substitutions, the arrow indicates the experimentally determined signal sequence cleavage site of IL-20, and underlines show the helical regions of IL-10. The location of conserved cysteines is indicated in bold type. (B) Chromosomal mapping of IL-20. IL-20 maps to human chromosome 1q32 and is tightly linked to IL-10, IL-19 and MDA-7.

IL-20 and IL-19 contain six conserved cysteine residues in their mature protein sequence, in contrast to IL-10, which only has four. Protein crystallographic analysis of IL-10 indicates that it forms an intercalating dimer (Zdanov et al., 1995). Threading the IL-20 sequence onto the human IL-10 structure yielded predictions about the cysteine pairing and oligomerization of IL-20. By analogy with the two disulfide bonds in IL-10, the pairings Cys33-Cys126, and Cys80-Cys132 in IL-20 can be inferred. However, residues Gln81 and Asn134 in IL-10, corresponding to Cys81 and Cys134 in IL-20, have a separation of 11.6 Å. IL-10, which forms an intercalating dimer, contains Asn116 in a potential flexible hinge region between the core of one monomer and the helix contributed by the other monomer. If Cys81 and Cys134 in IL-20 were disulfide bonded, the hinge region would be brought into close contact with the amino-terminus of helix B, leading to the prediction that IL-20 does not form an intercalating dimer. Analysis of recombinant human IL-20 produced in BHK and in baculovirus cells supports this prediction.

IL-20 Maps to a Cytokine Cluster

Radiation hybrid mapping was used to map IL-20 to human chromosome 1q32. Chromosomal localization of mouse IL-20 showed that it maps to the syntenic region on mouse chromosome 1. Since IL-10 maps to the same region (Eskdale et al., 1997), we mapped the other four IL-10 family members. MDA-7 and IL-19 also map to chromosome 1q32 (Figure 1B) whereas AK-155 and IL-TIF do not (Knappe et al., 2000 and data not shown). More detailed analysis using P1 artificial chromosome (PAC) clones allowed us to deduce the gene order on chromosome 1 as *IL-10*, *IL-19*, *IL-20*, *MDA-7* and to localize these genes within a 195 kb region (Figure 1B). These mapping studies indicate that four members of the IL-10 family comprise a cytokine cluster.

IL-20 Transgenic Mice Exhibit Aberrant Keratin Expression in Skin

Both human and mouse IL-20 were overexpressed in TG mice using a variety of promoters. The liver-specific mouse albumin (ALB) promoter (Pinkert et al., 1987), directing expression of human IL-20, was used initially in an attempt to achieve circulating levels of protein. Subsequent studies were conducted using the keratin 14 (K14) promoter (Vassar and Fuchs, 1989), which primarily targets expression to the epithelium; the mouse metallothionein-1 (MT-1) promoter (Palmiter et al., 1993), which gives a broad expression pattern; and the EμLCK

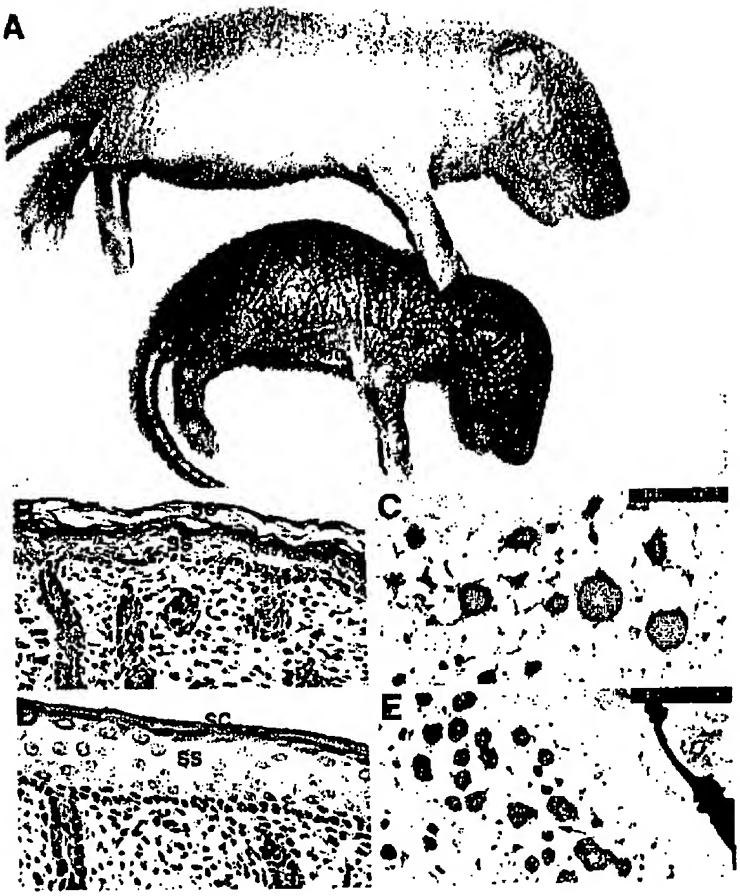


Figure 2. Skin Phenotype in IL-20 Transgenic Mice

(A) Shiny, wrinkled skin and smaller size in the IL-20 TG pup (bottom) compared to age-matched NTG littermate (top). (B) H&E-stained skin section (40 ·) from a 1-day-old NTG control mouse. SC stratum corneum, SS stratum spinosum. (C) Electron micrograph (EM) of the skin from a 1-day-old NTG mouse (16,000 ·). Note that the keratohyaline granules (white asterisk) are electron-dense. (D) H&E-stained skin section from a 1-day-old TG mouse with the ALB promoter driving mouse IL-20 (40 ·). The SC is more compact and the SS is thicker than in the age-matched control. (E) EM from a 1-day-old TG mouse with the ALB promoter driving human IL-20 (16,000 ·). The keratohyaline granules (white asterisk) are moth-eaten and scattered mitochondria (arrow) contain lipoid inclusions.

promoter (Iritani et al., 1997), which drives expression in cells of the lymphoid lineage. Similar results were obtained in all four cases, possibly because these promoters all give rise to circulating IL-20.

In all cases, TG pups expressing the IL-20 transgene were smaller than non-TG (NTG) littermates, had a shiny appearance with tight, wrinkled skin, and died within the first few days after birth (Figure 2A). Pups had milk in their stomachs indicating that they were able to suckle. These mice had swollen extremities, tail, nostril, and mouth regions and had difficulty moving. In addition, the mice were frail, lacked visible adipose tissue and had delayed ear and toe development. Low expression levels in liver (less than 100 mRNA molecules/cell) were sufficient for both the neonatal lethality and skin abnormalities. TG mice without a visible phenotype either did not express the transgene, did not express it at detectable levels, or were mosaic.

Histologic analysis of the skin of the IL-20 TG mice showed a thickened epidermis, hyperkeratosis and a compact stratum corneum compared to NTG littermates (Figures 2B and 2D). Serocellular crusts (scabs) were observed occasionally. Electron microscopic (EM) analysis of skin from TG mice showed intramitochondrial lipoid inclusions, mottled keratohyaline granules, and relatively few tonofilaments (Figures 2C and 2E) similar to that observed in human psoriatic skin (Gijbels et al., 1995). However, immune infiltrates commonly found in human psoriatic skin were not observed in the IL-20 TG mouse skin. In addition, many of the TG mice had apoptotic thymic lymphocytes (data not shown). No other abnormalities were detected by histopathological analysis. These histological and EM results support and extend the observed gross skin alterations.

Immunohistochemical analysis of epidermal markers was utilized to determine whether normal skin differentiation had taken place in the TG mice. These studies were performed on TG mice expressing IL-20 from the K14 promoter. Positive staining for K5 and K14 was detected in the suprabasal layer of the epidermis in addition to the expected basal layer, while staining for the hyperproliferation marker K6 in the suprabasal layer was detected in TG but not in NTG littermate skin (Figure 3). In contrast, the staining pattern for K1 (suprabasal), filaggrin (granular), and loricrin (upper spinous, granular) did not differ in the two groups of mice (Figure 3 and data not shown). Similar immunohistochemical results were obtained in TG mice expressing IL-20 from the ALB promoter (data not shown). Therefore, overexpression of IL-20 in TG mice results in aberrant expression of several keratins indicative of altered epidermal differentiation.

IL-20 TG mouse skin has a thickened epidermis and expresses K6, both suggestive of hyperproliferation. To assay directly for hyperproliferation, PCNA immunohistochemical staining was performed on skin from TG mice expressing IL-20 from the ALB and K14 promoters,

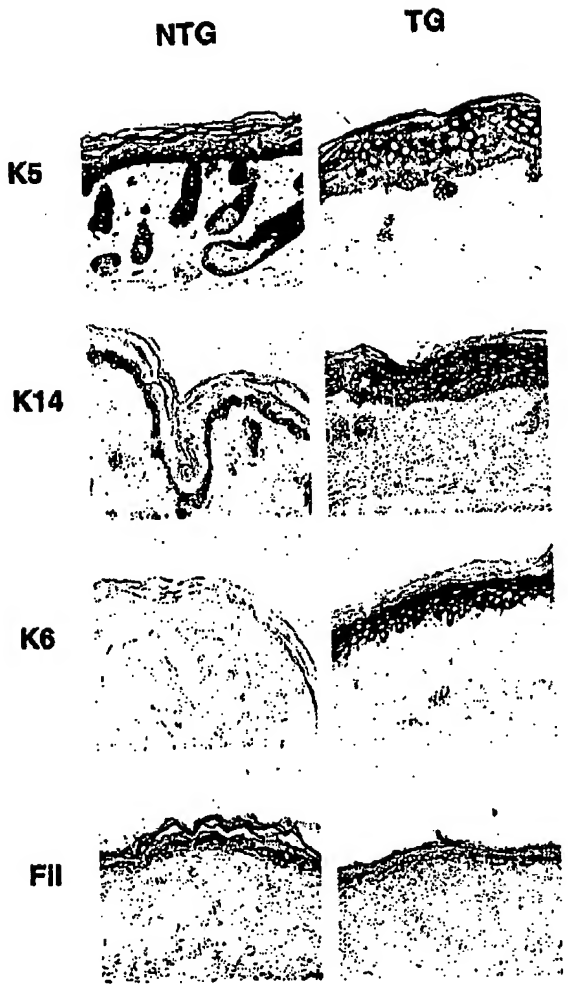


Figure 3. Aberrant Expression of Keratin Proteins Demonstrated by Immunohistochemical Analysis

K5 and K14 were detected in the basal layer of the epidermis in NTG mice. Similar sections obtained from TG mice derived from mouse IL-20 expressed from the K14 promoter show positive staining in the suprabasal layer of the epidermis as well. Also shown is positive staining for K6 in the suprabasal layer in TG mouse skin but not in NTG mouse skin. Filaggrin (Fil) staining was similar for both TG and NTG mouse skin (20×).

and from their NTG littermates. Staining in the basal skin layer was observed in NTG mice as expected, since this is the skin layer where proliferation normally takes place. However, in TG mouse skin, PCNA staining was observed in both the basal and suprabasal layers of the epidermis (data not shown). We conclude that hyperproliferation occurs in the skin of IL-20 TG mice.

Identification of a Heterodimeric IL-20 Receptor

Because IL-20 is homologous to IL-10, we hypothesized that the IL-20 receptor might share structural similarity with the heterodimeric IL-10 receptor. Therefore, we tested eight different class II cytokine receptors alone or in pairwise combinations in an IL-20 binding assay: IL-10Rα, IL-10Rβ (CRF2-4), interferon-αR1, interferon-αR2, interferon-γR1, and three orphan receptors zcytor7, zcytor11 (reported to be an IL-TIF receptor subunit; Xie et al., 2000), and DIRS1 (Parham et al., 1999). Candidate class II receptors were selected based on their expression in the human keratinocyte cell line HaCaT, previously determined to be responsive to IL-20. These receptors were transiently expressed in COS7 cells, which were then assayed for their ability to bind biotin-labeled IL-20 protein. None of the single receptor transfectants had detectable IL-20 binding. Only one pairwise combination of receptor subunits, zcytor7 (designated IL-20Rα) and DIRS1 (designated IL-20Rβ), gave rise to transfectants with positive IL-20 binding. Therefore, these proteins comprise a heterodimeric IL-20 receptor, structurally similar to the IL-10 receptor.

The specificity and affinity of IL-20 for its receptor was determined using BHK cells stably transfected with IL-20Rα, IL-20Rβ or both receptor subunits. Binding assays using radiolabeled ligand demonstrated that IL-20 bound to BHK transfectants expressing both IL-20Rα and IL-20Rβ, but not to untransfected cells nor to transfectants expressing either receptor subunit alone (Figure 4A). Binding of ^{125}I-labeled IL-20 was eliminated in the presence of 100-fold excess of unlabeled IL-20 but not in the presence of 100-fold excess of the unrelated cytokine, IL-21 (HUGO symbol for zalpha11 ligand; Novak et al., 2000). The binding data revealed 88,000 IL-20 receptors per cell with a binding affinity (K_d) of ~1.5 nM (Figure 4B).

IL-20 Binding Activates STAT3 in the HaCaT Keratinocyte Cell Line

As shown above, IL-20 binds cell lines transfected with both subunits of its receptor. However, these cell lines overexpress the IL-20 receptor relative to its normal level and their relevance to the physiological role of IL-20 is unclear. The human HaCaT keratinocyte cell line, which expresses endogenous IL-20Rα and IL-20Rβ, was used to examine IL-20 signal transduction in a biologically relevant cell type. HaCaT cells were infected with recombinant adenovirus containing a reporter construct to allow detection of intracellular signaling. The construct consists of the firefly luciferase gene driven by promoter/enhancer sequences comprised of the serum response element (SRE) and signal transducers and activators of transduction elements (STATs). This assay system detects productive ligand-receptor interactions and indicates possible downstream signal transduction components involved in receptor activation. Treatment with IL-20 alone resulted in a dose-dependent increase in luciferase activity with a half-maximal response occurring at ~2.3 nM (Figure 5A). Subsequent luciferase reporter assays using adenovirus vectors containing only the SRE element or only the STAT elements produced detectable reporter activation only through STATs (data not shown).

To determine if other cytokines act in concert with IL-20, HaCaT cells were treated with IL-20 alone or in combination with a single submaximal dose of EGF, IL-1β, or TNFα. These three proteins were chosen based on a screen of twelve cytokines and growth factors for activation of luciferase reporter constructs. Robust re-

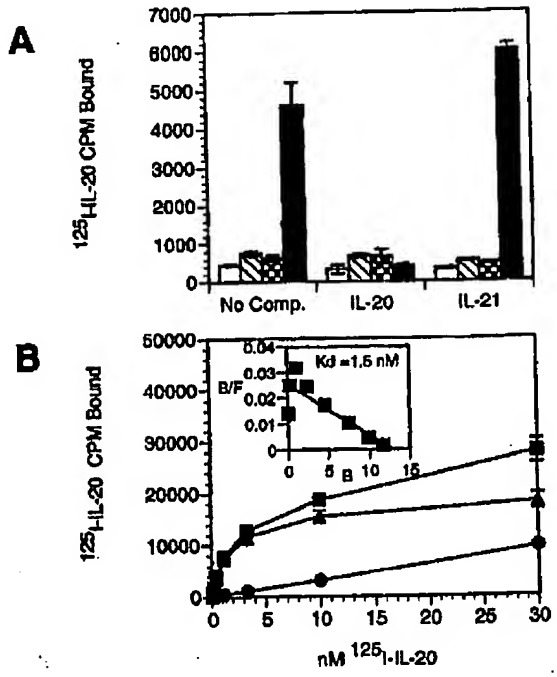


Figure 4. IL-20 Binds to the IL-20 Heterodimeric Receptor

(A) ^{125}I-IL-20 binds to transfected BHK cells expressing IL-20Rα and IL-20Rβ (solid bars), and is blocked by a 100-fold excess of IL-20 but not by IL-21. In contrast, ^{125}I-IL-20 does not bind to non-transfected BHK cells (open bars), to transfected BHK cells expressing IL-20Rβ alone (cross-hatched bars) or to IL-20Rα alone (tiled bars). (B) Saturation curve for binding of ^{125}I-IL-20 to transfected BHK cells expressing IL-20Rα and IL-20Rβ. The curves represent total binding (solid squares); nonspecific binding in the presence of 100-fold excess unlabeled IL-20 (solid circles); and specific binding determined by subtraction of nonspecific from total binding (solid triangles). A Scatchard transformation of the specific binding data is shown in the insert.

sponses were observed with EGF, IL-1β, TNF-α, and TGF-α, while KGF gave a response similar in magnitude to IL-20. The other seven cytokines, including IL-10, were inactive in this assay. In the presence of EGF, IL-1β, or TNF-α, IL-20 treatment resulted in a dose-dependent increase in luciferase activity (Figure 5A and data not shown). IL-20 in combination with IL-1β results in a half-maximal response at ~0.5 nM, about 5-fold lower than with IL-20 alone. In addition, activation of the reporter gene is detectable at 0.1 nM IL-20, a dose that is at least 10-fold lower than the IL-20 dose required alone.

BHK cells transfected with IL-20Rα, IL-20Rβ, or both receptor subunits were used to determine whether receptor pairing was required for IL-20 stimulation of STAT-luciferase. As was the case with binding assays, only cells transfected with both receptor subunits responded to IL-20 and did so with a half-maximal response of 6.7 pM (Figure 5B). We note that the IL-20 concentration for the half-maximal response in BHK cells is 400-fold lower than that for half-maximal response in HaCaT cells. It is likely that a lower concentration of IL-20 is needed for half-maximal response in BHK cells, as compared to HaCaT cells, due to higher receptor levels in the BHK IL-20 receptor transfectants.

It is well established that ligand binding to class II cytokine receptors, such as IL-10R, leads to activation of JAK kinases, phosphorylation of STATs and their subsequent translocation into the nucleus, where they activate transcription of target genes. A nuclear translocation assay was used to identify STAT proteins involved in IL-20 action. Both HaCaT cells, with endogenous IL-20 receptors, and BHK cells transfected with IL-20Rα and IL-20Rβ were treated with IL-20 protein, and translocation of STAT3 and STAT1 transcription factors from the cytoplasm to the nucleus was assayed by immunofluorescence.

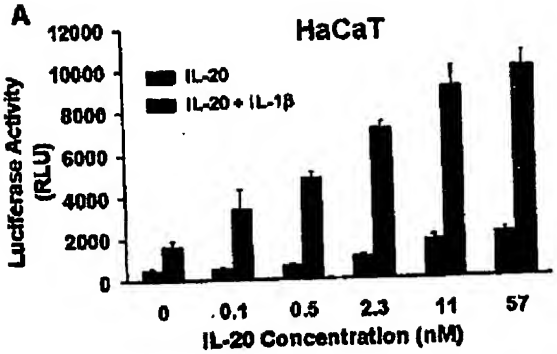
In unstimulated HaCaT cells, STAT3 staining was predominantly in the cytosol (Figure 5C). Treatment of HaCaT cells with IL-20 resulted in a distinct accumulation of STAT3 in the nucleus (Figure 5D). In contrast to STAT3 translocation, HaCaT cells treated with IL-20 did not show any detectable nuclear accumulation of STAT1 (data not shown).

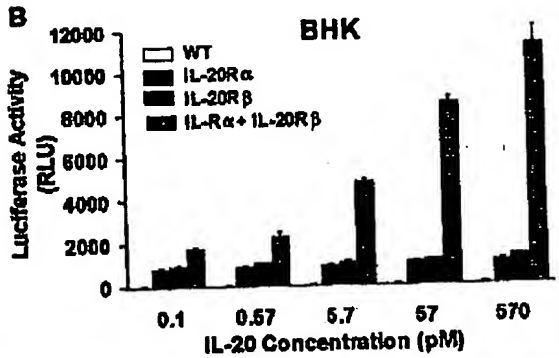
BHK cells transfected with IL-20Rα and IL-20Rβ were used to confirm that the IL-20 receptor was required for IL-20 stimulation of STAT3 nuclear translocation. In BHK cells lacking the IL-20 receptor, STAT3 remained cytosolic following treatment with IL-20 (Figure 5E). In contrast, in BHK cells transfected with the IL-20 receptor, STAT3 translocated to the nucleus in response to IL-20 (Figure 5F). Again, STAT1 remained cytosolic regardless of IL-20 treatment or IL-20 receptor expression (data not shown). We conclude that the IL-20 receptor is required for IL-20-mediated STAT3 activation.

To screen for potential downstream target genes for IL-20 activity, microarray analysis was performed on mRNA isolated from HaCaT cells treated with IL-20, IL-1, or IL-20 plus IL-1. Human cytokine, growth factor, and receptor genes were represented on the microarray. Expression of a number of genes increased 2- to 4-fold in response to IL-20 alone. In addition, a different, but overlapping, set of genes had higher expression in response to IL-20 + IL-1 than the sum of the individual cytokine responses (Experimental Procedures). A high percentage of these genes are involved in inflammation. RT-PCR analysis on three of these genes, *TNF-α*, *MRP-14*, and *MCP-1*, confirmed these results (data not shown). We conclude that the magnitude of specific target gene expression based on microarray and RT-PCR results correlated with those of reporter gene activation in HaCaT cells. IL-20 alone exhibited a modest increase in specific target gene expression and showed enhancement of the IL-1-induced expression of at least three genes known to be involved in inflammatory responses.

Expression of IL-20Rα and IL-20Rβ in Skin and Upregulation in Psoriasis

The expression pattern of IL-20Rα and IL-20Rβ in a variety of human tissues was determined by RT-PCR analysis. Both IL-20Rα and IL-20Rβ are most highly expressed in normal skin and testis, with lower expression in a variety of other normal tissues (Figure 6). In addition, IL-20Rα mRNA appears to be expressed in many tissue types where IL-20Rβ expression is undetectable under similar conditions. Most significant for the present study





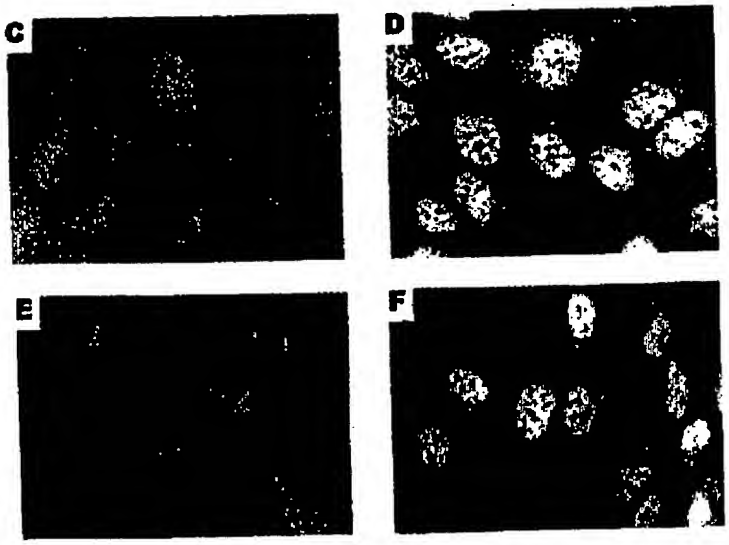


Figure 5. IL-20 Stimulates a Signal Transduction Pathway through STAT3

Panels (A) and (B) utilize luciferase reporter constructs to detect intracellular signaling. (A) HaCaT cells were infected with an adenovirus construct containing SRE and STAT promoter elements driving expression of luciferase. Treatment of HaCaT cells with IL-20 alone results in a dose-dependent increase in luciferase activity, which is dramatically enhanced by the addition of 0.1 nM IL-1β. (B) BHK cells were stably transfected with STAT promoter elements driving expression of luciferase. Treatment of BHK cells expressing IL-20Rα and IL-20Rβ with IL-20 results in a dose-dependent increase in luciferase activity. Panels (C)–(F) assay the translocation of STAT3 transcription factor from the cytoplasm to the nucleus by immunofluorescence. STAT3 staining was predominantly in the cytosol in unstimulated HaCaT cells (C), whereas treatment of HaCaT cells with IL-20 resulted in a distinct nuclear accumulation of STAT3 (D). BHK cells lacking the IL-20 receptor had cytosolic STAT3 following treatment with IL-20 (E), whereas BHK cells transfected with IL-20Rα and IL-20Rβ, had STAT3 translocation to the nucleus in response to IL-20 (F).

is that IL-20Rα and IL-20Rβ are both expressed in skin, where they are involved in IL-20 binding and activation of signal transduction.

We hypothesized that the IL-20 receptor may have altered regulation in skin diseases. To test this hypothesis, in situ hybridization was performed on skin samples from seven psoriasis patients and three patients with normal skin. All seven psoriatic skin samples had high levels of IL-20Rα and IL-20Rβ mRNA in keratinocytes whereas normal skin samples had minimal to undetectable levels of either receptor subunit mRNA (Figures 7A–7F). The difference between these in situ hybridization results and the RT-PCR results described above for normal skin may reflect the higher sensitivity of the RT-PCR assay. In some of the psoriatic skin samples, positive signals were also observed for both IL-20 receptor subunits in mononuclear immune cells and in endothelial cells in a subset of vessels (Figures 7G and 7H). Therefore, both IL-20Rα and IL-20Rβ are expressed in keratinocytes, immune cells, and endothelial cells, the

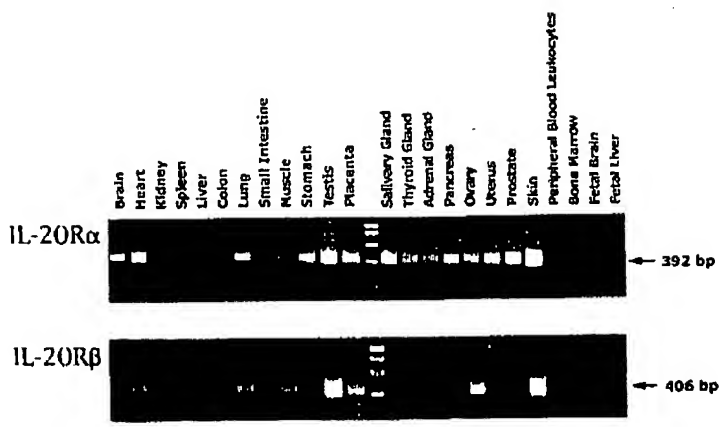


Figure 6. Expression of IL-20Rα and IL-20Rβ in Human Tissues

RT-PCR analysis of IL-20Rα and IL-20Rβ was performed using a panel of 24 human tissues. Both receptor subunit mRNAs are highly expressed in skin and testis.

major cell types thought to interact in psoriasis (Bos and De Rie, 1999; Karasek, 1999).

Discussion

IL-20 stimulates signal transduction in the human keratinocyte HaCaT cell line, supporting a direct action of this novel ligand in skin. In addition, IL-1β, EGF, and TNF-α, proteins known to be active in keratinocytes and to be involved with proliferative and proinflammatory signals in skin, enhance the response to IL-20. IL-20 may be a cytokine that exhibits its most potent effects in combination with other factors, similar to the role that stem cell factor and flt-3 ligand play in the hematopoietic system (Koller et al., 1996). In both HaCaT and BHK cells expressing the IL-20 receptor, IL-20 signals through STAT3 as does IL-10 (Kotenko and Pestka, 2000). IL-10 also activates STAT1 to some extent, which was not observed for IL-20. Future experiments will be directed at defining additional components of the IL-20 signal transduction pathway and at understanding the role of its inflammation-related target genes.

Colocalization of IL-20 with three other IL-10 family members identifies the 1q32 region as a cluster of cytokine genes. Clustering of family members may be the result of gene duplication with subsequent divergence of function and regulation. The known activities for the IL-10 family members in this cluster reflect this functional divergence. IL-10 has anti-inflammatory activity in skin due to inhibition of proinflammatory cytokine synthesis. Consistent with this observation, expression of both IL-10 and the IL-10 receptor are decreased in psoriatic skin (Michel et al., 1997; Asadullah et al., 1998). In contrast, expression of both IL-20 receptor subunits is increased in psoriatic skin. MDA-7 expression is elevated in melanoma cells and is thought to have antitumor activity (Jiang et al., 1996), while the actions of IL-19 have not been described. Further experimentation is needed to elucidate the functions of the new members of this IL-10 family to more comprehensively understand their similarities and differences.

Two orphan class II cytokine receptors, both of which are expressed in skin, were identified as IL-20 receptor subunits. Both IL-20 receptor subunits are required for ligand binding, distinguishing their role from that of subunits in the four other known class II cytokine receptors (Domanski and Colamonici, 1996; Pestka et al., 1997; Xie et al., 2000). For example, in the IL-10 heterodimeric receptor, the IL-10Rα subunit is sufficient for high-affinity ligand binding, while IL-10Rβ is required for signaling (Kotenko et al., 1997). IL-20Rα and IL-20Rβ are also coexpressed in a number of human tissues besides skin, suggesting additional target tissues for IL-20 action. Additionally, we have detected IL-20Rα mRNA, but not IL-20Rβ mRNA, in several human tissues suggesting that IL-20Rα may partner with other class II receptor subunits. However, unlike the class I cytokine receptor common signaling subunits β_c, gp130, and IL2Rγ, there is only one example of a shared receptor subunit (CRF2-4) in the class II receptor family (Dumoutier et al., 2000b; Xie et al., 2000). It is also possible that the IL20Rα/IL20Rβ heterodimeric receptor acts as a receptor for other IL-10 family members. We conclude that the IL-20 heterodimeric receptor is structurally similar to other class II cytokine receptors and is expressed in skin where we have demonstrated activity of the IL-20 ligand.

Two lines of evidence suggest a role for IL-20 and its receptor in psoriasis. This multigenic skin disease is characterized by increased keratinocyte proliferation, altered keratinocyte differentiation, and infiltration of immune cells into the skin (Bos and De Rie, 1999). The first line of evidence for a role of IL-20 in psoriasis is that the observed hyperkeratosis, thickened epidermis, and proliferation in the suprabasal layer in the TG mice that resemble human psoriatic abnormalities. Changes detected in the epidermal protein expression in IL-20 TG mice include the presence of the basal K5 and K14 in both the basal and suprabasal layers, similar to the changes observed in human psoriatic skin (Sun et al., 1985; Castellijns et al., 1999). IL-20 TG mice also express the hyperproliferative-associated K6, present in human psoriatic but not normal skin. Similar skin abnormalities have been reported in TG mouse models expressing KGF, TGF-α, and interferon γ from epidermal-specific promoters (Vassar and Fuchs, 1991; Guo et al., 1993; Carroll et al., 1997). No TG mouse model demonstrating all aspects of human psoriasis exists. For example, immune infiltrates were not observed in the IL-20 TG mice. In addition, the ultrastructural features of the IL-20 TG

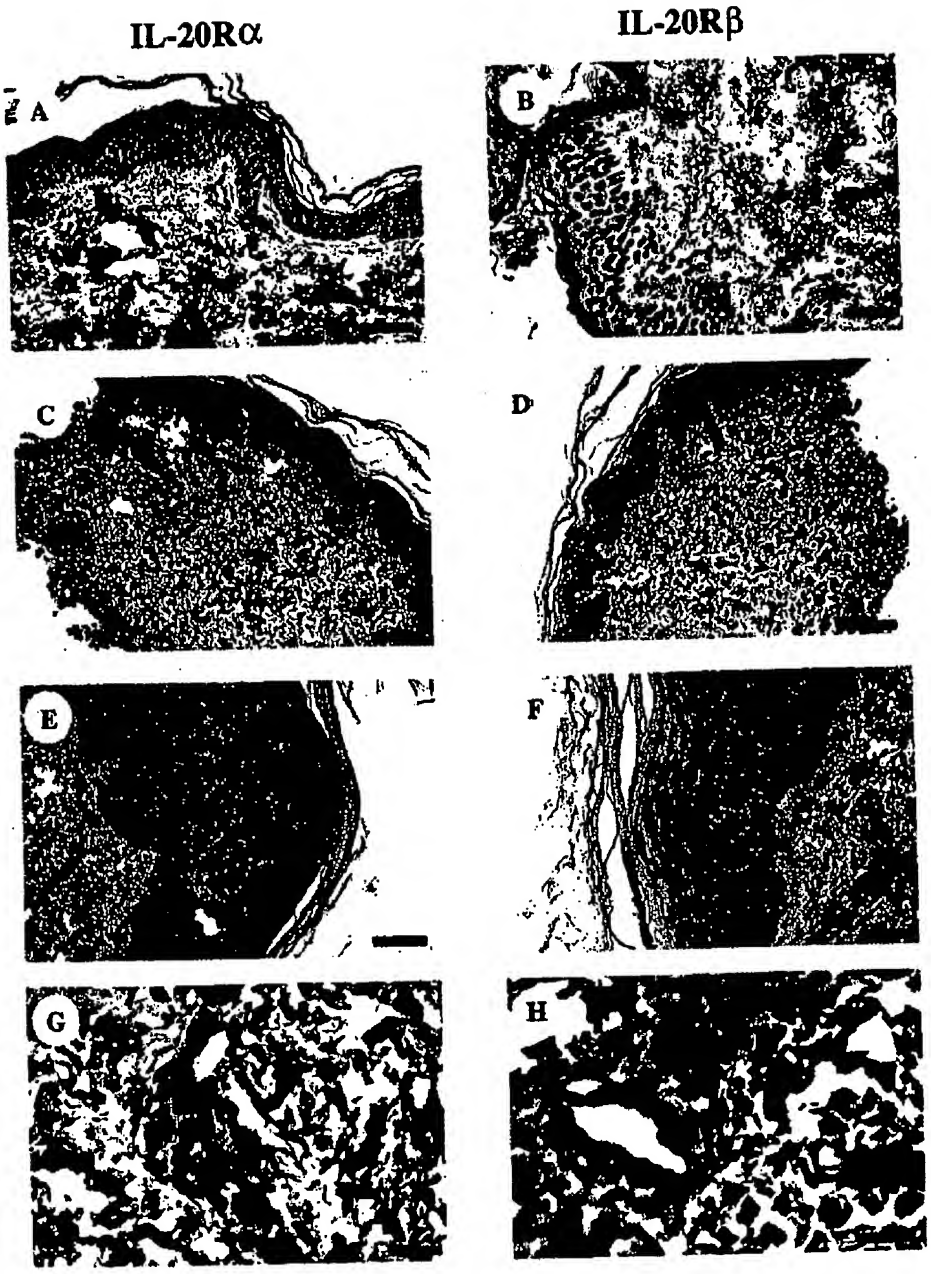


Figure 7. In Situ Hybridization Demonstrates IL-20Rα and IL-20Rβ mRNA Upregulation in Psoriasis

IL-20Rα mRNA expression is shown in (A), (C), (E), and (G), whereas IL-20Rβ mRNA expression is shown in (B), (D), (F), and (H). Normal human skin shows minimal to undetectable levels of both IL-20Rα and IL-20Rβ mRNA (A and B). In contrast, increased staining for both receptor subunits is observed in human psoriatic skin (C–H). Upregulation of both receptor subunit mRNAs is detected in keratinocytes (E and F), as well as in endothelial and immune cells (G and H). Scale bar indicates: (A and B) = 1 mm; (C and D) = 2 mm; (E and F) = 0.5 mm; and (G and H) = 0.2 mm.

mouse skin have been found in other hyperproliferative diseases of the epidermis. Decreased numbers of tonofilaments, thought to be related to defective keratinization, are a striking feature of human psoriasis (Jahn et al., 1988). Intramitochondrial inclusions have been found in both chemically induced and naturally occurring hyperplastic skin conditions in mice (Gijbels et al., 1995). Finally, mottled keratohyaline granules were found in neonatal TGFα TG mice that had thickened skin due to epidermal proliferation (Vassar and Fuchs, 1991). We conclude that IL-20 TG mice appear to exhibit many of the characteristics observed in human psoriasis.

A second line of evidence that implicates the IL-20 receptor in psoriasis is that both IL-20Rα and IL-20Rβ mRNA are markedly upregulated in human psoriatic skin compared to normal skin. Both IL-20 receptor subunits are expressed in keratinocytes throughout the epidermis and are also expressed in a subset of immune and endothelial cells. We propose that increased expression of an activated IL-20 receptor may alter the interactions between endothelial cells, immune cells and keratinocytes, leading to dysregulation of keratinocyte proliferation and differentiation.

A crucial step in understanding the function of a novel cytokine is the identification and characterization of its cognate receptor. We have successfully used a structure-based approach to isolate a novel interleukin and defined skin as a target tissue, which ultimately led to the isolation of its receptor. We demonstrate that IL-20 binds its receptor on keratinocytes and stimulates a STAT3-containing signal transduction pathway. The effect of IL-20 on skin differentiation in TG mice, together with the upregulation of the IL-20 receptor subunits in psoriasis, pave the way for future studies on this ligand/receptor pair in inflammatory skin diseases.

Experimental Procedures

Bioinformatics Analysis

A novel "quasi-threading" algorithm, which inspects sequences for multiple profiles, was employed. Ungapped amino acid profiles of width n are two-dimensional matrices with dimensions n by 20 (Gribskov et al., 1987). Profiles are used to provide the score for amino acids in each position of a polypeptide segment of length n. Translated EST and other DNA database sequences were threaded using a protein structure–based profile linked by regions of variable length. Two profiles of width 15, which described a signal sequence and an amino-terminal amphipathic helix typical of helical cytokines with a variable region from 1 to 30 residues, were used.

Cloning of Mouse IL-20

Using a probe corresponding to the coding region of human IL-20, a mouse genomic lambda library (Clontech) was screened at low stringency, potential positives were identified and plaque purified to homogeneity, and subclones were generated for sequencing. The sequence of one subclone revealed two predicted exons homologous to human IL-20. Oligonucleotides were designed to this sequence and used for 5′ and 3′ RACE on mouse skin Marathon-ready cDNA, to generate the full-length coding sequence for mouse IL-20.

Chromosomal Mapping of IL-20

IL-20 was mapped to chromosome 1 on both the lower resolution GeneBridge 4 and the medium resolution Stanford G3 radiation hybrid (RH) mapping panels. Mouse IL-20 was mapped by PCR using the commercially available mouse T31 whole genome radiation hybrid (WGRH) panel (Research Genetics, Inc.) and IL-20 murine-specific sense and anti-sense primers. IL-10, IL-19, and MDA-7 were mapped by PCR using the commercially available version of the Stanford G3 Human/Hamster RH panel (Research Genetics, Inc.).

A PAC library and high-density array filters were obtained from Roswell Park Cancer Institute and were hybridized with probes from the coding regions of human IL-19 and MDA-7. Four PAC clones were identified in this screen. Two additional clones (63B3 and 63H3) were identified in a library from Genome Systems, using a PCR screen with IL-10 primers. Inserts were sized using NotI digestion to excise inserts, followed by pulsed-field gel electrophoresis. To determine gene order, PACs were analyzed using PCR (IL-19, MDA-7, and IL10) and hybridization (IL-20, MDA-7). PAC 141G11 was negative by PCR for MDA-7, but was positive by hybridization, indicating that the PAC contains part of the gene but is missing the MDA-7 primer region.

IL-20 Transgenic Mice

Both human and mouse IL-20 cDNA coding sequences were amplified by PCR and cloned into plasmids containing different promoters. Introns from rat insulin II, SV40 T-antigen, or human growth hormone were cloned either 5′ or 3′ of the IL-20 cDNA to improve expression. Primers contained a consensus translation initiation site upstream of the ATG. All plasmids used contain a 650 bp human growth hormone (hGH) poly A sequence 3′ of the coding sequence.

One half centimeter tail snips were isolated from pups (C57/Bl6 X C3H F2 hybrid mice) resulting from microinjection of the linearized expression cassette DNA. Genomic DNA was isolated using the DNAeasy kit (Qiagen). Genotyping was performed by PCR using three sets of primers against specific regions of the TG plasmids: hGH 3′ UTR, flanking regions for the IL-20 cDNA insert, and an endogenous mouse gene.

Mouse tissues were dissected, stored in RNAlater (Ambion) and total RNA was isolated using the RNAeasy kit (Qiagen). Expression analysis of IL-20 TG mRNA was performed using the Taqman RT-PCR assay (Perkin Elmer) with primers against the hGH 3′ UTR.

Histological Analysis

Tissues for light microscopy were collected in 10% neutral buffered formalin, routinely processed, sectioned at 5 μm and stained with hematoxylin and eosin. Skin sections for electron microscopy were collected into paraformaldehyde-glutaraldehyde, routinely processed, and stained with uranyl acetate (Sheehan and Hrapchak, 1980).

Immunohistochemistry for Epidermal Proteins

Carnoy's fixed skin from the backs of normal and TG (newborn to day 3) mice were sectioned for immunohistochemistry. Polyclonal antibodies to mouse keratins 1, 5, 6, 10, and 14 as well as polyclonal antibodies to mouse loricrin, involucrin and pro/filaggrin (Berkeley Antibody Company) were followed by secondary biotinylated anti-rabbit IgG, amplified with immunoperoxidase (Vectastain ABC kit, Vector Laboratories), and visualized with diaminobenzidine as chromagen. PCNA immunohistochemistry was performed as described by Dietrich (1993) on skin from TG mice expressing IL-20 from the ALB and K14 promoters, and from their NTG littermates.

Recombinant IL-20

IL-20 cDNA was generated by PCR and inserted into the pZP9NEE mammalian expression vector containing the MT-1 promoter or the baculovirus expression vector pZBV3L (a modification of an Invitrogen vector) containing the basic protein promoter. NEE is an N-terminal sequence tag with the sequence EYMPMEGS. The pZP9NEE-IL-20 vector was transfected into BHK570 cells by lipofectamine and selected in DMEM + 5% FBS with 1 μM methotrexate. Supernatants from resistant cells were analyzed for protein and high expressing clones were scaled up in cell factories. Protein was purified from filtered BHK570 culture supernatants by EE monoclonal antibody affinity chromatography (Grussenmeyer et al., 1985).

Receptor Binding Assays

A modification of the "secretion trap" (Davis et al., 1996) was performed on COS7 cells transfected with candidate class II cytokine receptor cDNAs.

Cell Culture and Transfections

HaCaT cells (Boukamp et al., 1988), were obtained from Dr. N. Fusenig. BHK570 cells are deposited at ATCC (CRL-10314). Stable BHK cell lines were generated by selection in puromycin (IL-20-Rα) and zeocin (IL-20-Rβ).

Binding Assays

Human IL-20 was iodinated using Iodobeads (Pierce). For competition binding assays, 250 pM ^{125}I-IL-20 was used with or without 25 nM competitor proteins. For saturation binding, a 30 nM to 13.7 pM dilution series of ^{125}I-IL-20 was used, with or without a 100-fold excess of

unlabeled IL-20 for determination of specific binding. Scatchard analysis was done using Delta Graph 4.0 (Delta Point, Inc.).

Luciferase Assays

CMV promoter/enhancer and SV40 pA sequences were removed from pACCMV.pLpA (Becker et al., 1994) and replaced with a linker containing KpnI and HindIII sites. The STAT/SRE-driven luciferase reporter cassette was excised from vector KZ136 (Poulsen et al., 1998) as a KpnI-HindIII fragment and inserted into the vector described above. Recombinant KZ136 adenovirus was produced by transfection with JM17 adenovirus into 293 cells (Becker et al., 1994). Plaque purified virus was amplified and used to infect cultured cells at 5–50 pfu/cell 12–36 hr before assay. Luciferase reporter assays were performed with a 4 hr induction time following treatment (Poulsen et al., 1998).

STAT Nuclear Translocation Assay

HaCaT or BHK cells were treated with IL-20, interferon-γ (positive control for STAT1 translocation), or interferon-α (positive control for STAT3 translocation) for 20 min and analyzed using STAT1 and STAT3 HitKit™ reagent kits (Cellomics, Inc). Cells were incubated with anti-STAT3 or anti-STAT1 antibody (rabbit polyclonal) for 1 hr, followed by incubation with secondary antibody (AlexaFluor® 488 conjugated goat anti-rabbit IgG) and Hoechst 33342 dye. Cells were examined on an inverted, fluorescent microscope and images recorded with a CCD camera. To generate a dose-response curve and calculate the IL-20 concentration for a half-maximal response, fields were scanned, images were acquired and analyzed by the ArrayScan® II system (Cellomics®, Inc.) using an image processing algorithm for cytosol-nuclear translocation (Ding et al., 1998).

Microarray Assays

HaCaT cells were grown to post-confluence and were switched to serum-free media for two days. Cells were then treated as follows for 4 hr: IL-1α (5 ng/ml), IL-1α (5 ng/ml) + IL-20 (1 μg/ml), IL-20 (1 μg/ml), or no cytokine addition. Total RNA was isolated by CsCl gradient centrifugation. Protocols for polyA+ enrichment, probe synthesis and hybridization to Atlas™ arrays were provided by Clontech. Array membranes were exposed on a phosphorimager screen overnight and analyzed using AtlasImage™ 1.0 software (Clontech).

Genes whose expression increased at least 2-fold with IL-20 addition (average fold increase): TNF-α (2.2) and MRP-14 (3.4). Genes with enhanced expression due to the combination of IL-20 and IL-1 (IL-20 alone, IL-1 alone, IL-20 + IL-1): MRP-14 (3.0, 12.2, 20.3) and MCP-1 (1.3, 32.4, 55.9).

RT-PCR Analysis on Human Tissue

RT-PCR was performed on Rapid-Scan human gene expression panels (Origene Technologies, Inc.). Oligonucleotide primers 5' TCAAACAGAACGTGGTCCCAGTG 3' and 5' TCCGAGATATTGAGG GTGATAAAG 3' were used to generate a 392 bp IL-20Rα fragment. Oligonucleotide primers 5' GCTGGTGCTCACTCACTGAAGGT 3' and 5' TCTGTCTGGCTGAAGGCGCTGTA 3' were used to generate a 406 bp IL-20Rβ fragment. PCR conditions were 94°C for 3 min followed by 35 cycles of 94°C/30 s, 64°C/30 s, 72°C/120 s, followed by 72°C for 5 min.

In Situ Hybridization

In situ hybridization probes were designed against human IL-20Rα and IL-20Rβ. Blastn searches against our databases, which include all known class II cytokine receptors, demonstrated that the probes were specific for their respective receptors. PCR products were used as templates for synthesis of digoxygenin-labeled antisense RNA probes. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Hybridization was carried out at 60°C with 50% formamide/2× SSC. The signals were amplified with two to three rounds of tyramide signal amplification (TSA *in situ* indirect kit, NEN) and visualized with Vector Red substrate kit (Vector Lab). Slides were counterstained with hematoxylin. All tissues were tested with positive control probes.

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GenBank Accession Numbers

The GenBank accession number for human IL-20 is AF224266 and for mouse IL-20 is AF224267. The human gene symbol *IL-20* was approved by the HUGO Nomenclature Committee.

IL-19 was discovered by Human Genome Sciences, Inc. and is described in the International patent application WO 98/08870. It was independently discovered at ZymoGenetics and assigned GenBank accession number AF192498.

The three orphan class II cytokine receptors are zcytor7 and zcytor11 (ZymoGenetics) and DIRS1 (Schering). The GenBank accession number for zcytor7 is AF184971 described in U.S. Patent No. 5,945,511 (1999). The GenBank accession number for zcytor11 is X24379, described in international patent application publication number WO 2000/39161. Human DIRS1 was described in international patent application publication number WO 99/46379 (1999) by Schering Corporation. The human gene symbols *IL-20RA* (encoding IL-20Rα) and *IL-20RB* (encoding IL-20Rβ) were accepted by the HUGO Nomenclature Committee.